**Ablation of calcitonin/calcitonin gene-related peptide-α impairs fetal magnesium but not calcium homeostasis**

Kirsten R. McDonald, Neva J. Fudge, Janine P. Woodrow, James K. Friel, Ana O. Hoff, Robert F. Gagel, and Christopher S. Kovacs

1Faculty of Medicine - Endocrinology, Memorial University of Newfoundland, St. John’s, Newfoundland A1B 3V6; 2Department of Biochemistry and Pediatrics, Memorial University of Newfoundland, St. John’s, Newfoundland A1B 3X9, Canada; and 3University of Texas MD Anderson Cancer Research Center, Houston, Texas 77030

Submitted 16 January 2004; accepted in final form 18 March 2004

**Fetal mineral homeostasis is regulated differently from adult homeostasis, reflecting the unique needs of the developing fetus, which include maintaining serum mineral concentrations higher than in the mother, actively transporting mineral across the placenta, and mineralizing the developing skeleton (23, 27). Parathyroid hormone (PTH) and PTH-related protein (PTHrP) have been shown to play interlocking roles in the regulation of fetal blood calcium, placental calcium transfer, and formation of the fetal skeleton (20, 24, 28, 29). Vitamin D, calcitriol, and its receptor have been shown in a number of experimental models to be relatively unimportant for fetal mineral homeostasis (see review in Ref. 23), unlike in the adult, where these elements are critically required.**

The role of calcitonin in fetal mineral metabolism has not been extensively examined. Calcitonin is expressed by human thyroidal C cells early in gestation (33), and it circulates in the fetal circulation at levels that are higher than in the mother (23, 27). With respect to serum calcium regulation, Garel and colleagues established that pharmacological administration of calcitonin (16) or of calcitonin antiserum (15) altered the serum calcium of fetal rats in predictable and opposite directions. However, Care et al. (9) found that fetal thyroidectomy with subsequent thyroxine replacement did not affect the fetal blood calcium in sheep, indicating that the physiological amounts of calcitonin derived from the fetal thyroid may not be required to maintain a normal serum calcium.

With respect to placental calcium transfer, the potential role of calcitonin was indirectly examined by Barlet and colleagues in two reports. Skeletal mineral content was reduced in thyroidectomized fetal sheep that received thyroxine but not calcitonin supplementation (4), and pharmacological doses of calcitonin decreased PTHrP-stimulated increases in fetal skeletal calcium content (5). However, placental calcium transfer was not directly measured in those two studies; instead, skeletal ash weight and mineral content were measured several days after initiation of treatment. The sole study that directly measured placental calcium transfer was that of Care et al. (9), in which fetal thyroidectomy with subsequent thyroxine replacement did not alter placental calcium transfer in fetal sheep as determined in a placental perfusion model.

The previously cited studies did not adequately examine the physiological role of calcitonin in fetal mineral homeostasis, because pharmacological manipulations were done, and because the surgical removal of the thyroid did not create a completely calcitonin-deficient state. It has since been established that calcitonin is also produced in the central nervous system, late pregnant and lactating breast, uterus, and placenta (3, 8, 25). In particular, the presence of calcitonin and its receptor in the placentas of humans and mice indicates that it may have a role in some aspect of placental function (25).

To examine a model that is completely deficient in physiological amounts of calcitonin, we have utilized the calcitonin/calcitonin gene-related peptide-α (Ct/Cgrp) gene knockout model. Cgrpα is an alternative splice product of the calcitonin gene; a second gene produces Cgrpβ. Therefore, Ct/Cgrp null
mice completely lack calcitonin but still produce CGRP through the CGRPβ gene. Ablation of Ct/Cgrp in mice has been shown to result in a phenotype characterized by increased bone mass, increased bone formation, and preservation of bone mass during acute estrogen withdrawal from ovariec-tomy (18), but its role in fetal mineral homeostasis had not yet been reported.

In this study, we tested the hypothesis that calcitonin is neither required for the regulation of fetal serum calcium and normal skeletal development and mineralization in utero. We utilized the Ct/Cgrp null mice as a model for absence of calcitonin, and we contrasted the phenotype of these fetal mice with that of their wild-type (wt) and heterozygous (Ct, CGRP, and we contrasted the phenotype of these fetal mice together.

Animal husbandry. Ct/Cgrp gene knockout mice were obtained by targeted disruption of the murine gene in embryonic stem cells, as previously described (18). The original strain was backcrossed into Black Swiss (Taconic, Germantown, NY) for at least four generations, and the colony was maintained through breeding heterozygous mice 

\[ \text{Ct/Cgrp}^{+/+} \] 

and the colony was maintained through breeding heterozygous mice 

\[ \text{Ct/Cgrp}^{+/-} \] 

and the colony was maintained through breeding heterozygous mice 

\[ \text{Ct/Cgrp}^{--/--} \] 

sibling. The nature of the model meant that we tested for the effects of absence of both products of \text{Ct/Cgrp}, calcitonin and CGRPs. We found that absence of calcitonin and CGRPs results in modest abnormalities of magnesium metabolism but no alteration in calcium metabolism.

MATERIALS AND METHODS

Animal husbandry. Ct/Cgrp gene knockout mice were obtained by targeted disruption of the murine gene in embryonic stem cells, as previously described (18). The original strain was backcrossed into Black Swiss (Taconic, Germantown, NY) for at least four generations, and the colony was maintained through breeding heterozygous mice together. Ct/Cgrp\(^{+/+}\) males and females were mated to create pregnancies in which wt, Ct/Cgrp\(^{+/-}\), and Ct/Cgrp\(^{--/--}\) null fetuses were present. Ct/Cgrp\(^{+/+}\) males and Ct/Cgrp\(^{+/-}\) null females were also mated to yield pregnancies with Ct/Cgrp\(^{+/-}\) and Ct/Cgrp\(^{--/--}\) null fetuses. The Ct/Cgrp\(^{+/-}\) and Ct/Cgrp\(^{--/--}\) null females were first-degree relatives of each other. Mice were mated overnight; the presence of a vaginal mucus plug on the morning after mating marked gestational day 0.5. Normal gestation in these mice is 19 days. All mice were given a standard chow (1% calcium) diet and water ad libitum. All studies were performed with the prior approval of the Institutional Animal Care Committee of Memorial University of Newfoundland.

Genomic DNA was obtained from fetal tails, and genotyping was accomplished by PCR with three primers that were specific to the Ct/Cgrp gene sequence and the neomycin cassette, in a single-tube, 36-cycle PCR reaction utilizing a PTC-200 Peltier Thermal Cycler (MJ Research, Cambridge, MA). These primers utilized the following specific sequences: CAG GAT CAA GAG TCA CCG CT (CT-N). 36-cycle PCR reaction utilizing a PTC-200 Peltier Thermal Cycler

\[ \text{CAG GAT CAA GAG TCA CCG CT (CT-N)} \]

At the time of each cesarian section (day 17.5 or 18.5 of gestation), the number of viable fetuses present was counted. In separate studies, the number of live pups present at the time of weaning was counted to determine the postnatal litter size. This latter number is more variable than the number of fetuses, due to normal loss of pups in the neonatal period from factors such as maternal culling of the litter.

Chemical and hormone assays. Whole blood, serum, and amniotic fluid were collected using methods previously described (29). Ionized calcium was measured on whole blood using a Chiron Diagnostics 634 Ca\(^{++}\)/pH Analyzer (Chiron Diagnostics, East Walpole, MA). Serum PTH was measured on embryonic day 18.5 (ED 18.5) fetuses using a rodent PTH-(1-34) ELISA kit (Immutopics, San Clemente, CA); the stated detection limit of the assay was 1.6 pg/ml. Serum calcitonin was measured using an immunoradiometric assay (IRMA; Immunoexotics) developed to rat calcitonin, with sera from three or four fetuses pooled together to obtain sample volumes of 100 μl. These pooled samples were then diluted with zero standard (100) to meet the sample size requirements of the assay. No pooling was required for maternal samples. Total calcium, magnesium, and phosphorus were measured using photometric assays (Sigma-Aldrich, Oakville, ON).

Placental calcium transfer. This procedure has been described in detail elsewhere (28). Briefly, pregnant dams on ED 17.5 were given an intracardiac injection of 50 μCi ⁴⁵Ca and 50 μCi ⁵¹Cr-EDTA. Five minutes later, the dams were killed, and each fetus was removed. The radioactivity ratio of ⁴⁵Ca to ⁵¹Cr was determined for each fetus with a γ-counter and a liquid scintillation counter, respectively. The data were normalized to the mean ⁴⁵Ca/⁵¹Cr activity ratio of the Ct/Cgrp\(^{+/-}\) fetuses in each litter so that the results from different litters could be compared.

Fetal collection. For in situ hybridization and immunohistochemistry of fetal bones, whole fetuses (ED 17.5 or 18.5) were placed in 10% formalin after the first incision in the abdomen to prevent its gaseous expansion. For in situ hybridization of placentas, the maternal circulation was first perfused with phosphate-buffered saline followed by paraformaldehyde, after which the placentas were removed from the uterus and placed into fixative (29). After 12–24 h in the fixative, the limbs and placentas were removed and separately processed, embedded in paraffin, and cut into 5-μm sections.

Fetal ash and skeletal mineral assay. With methods previously described (24), intact fetuses (ED 18.5) were reduced to ash in a furnace (500°C × 24 h), and the ash was assayed for calcium and magnesium on a Perkin Elmer 2380 Atomic Absorption Flame Spectrophotometer. Because fetal size varied from litter to litter and would affect the individual measurements (large litter, smaller fetuses; small litter, larger fetuses), the data were normalized to the mean value of the heterozygotes within each litter. The heterozygotes were chosen as the baseline for this comparison because, on average, they accounted for 50% of the fetuses in a given litter.

Riboprobe and DNA probe labeling. For in situ hybridization, the plasmids were linearized with appropriate restriction enzymes and labeled with 125 μCi of ³²P-UTP by use of an SP6/T7 Transcription Kit (Promega/Fisher Scientific, Burlington, ON) and the appropriate polymerase. Unincorporated nucleotides were removed with the Nuclease Trap columns (Stratagene, La Jolla, CA).

Growth plate- and bone-related cDNAs included pro-α(I) chain of human type I collagen (6), pro-α(II) chain of rat type II collagen (22), H4 histone (39), and mouse type X collagen (1) (gifts of K. Lee); mouse osteocalcin (14), rat osteopontin (36), rat alkaline phosphatase (40), and murine cartilage matrix protein (matrilin 1) (2) (gifts of B. Lanske); rat PTHrP (21) (gift of H. M. Kronenberg); murine interstitial collagenase (17) and murine 92-kilodalton gelatinase (type IV collagenase or MMP-9) (38) (gifts of S. M. Krane).

Placental cDNAs used included murine calbindin-D28k (35) (gift of S. Christakos), human Ca²⁺/ATPase (30) (gift of R. Kumar), murine α-fetoprotein (gift of Margaret Baron), murine placental lactogen (19) and murine prolactin (34) (gifts of D. Linzer), and murine nodin (41) (gift of M. Kuehn).

In situ hybridization. In situ hybridization was performed on 5-μm tissue sections, as described previously (32). Hybridization was performed in a humidified chamber (16 h, 55°C) with the labeled riboprobe diluted 1:20 in the hybridization solution. Sections were successively washed, RNase treated, and dehydrated in graded ethanol (EtOH) series. An overnight exposure of the slides to plain X-ray film enabled an estimate of exposure time for the liquid emulsion step. Slides were then dipped into NTB-2 liquid emulsion, dried, stored in light-tight boxes, and kept at 4°C until developed (2–6 wk). The emulsion was developed using standard developer and fixer, and the sections were counterstained with hematoxylin-eosin.

All comparisons of wt with Ct/Cgrp null animals were made between tissues obtained from within the same litter, and which had been processed, embedded, and sectioned at the same time. All comparative sections were always hybridized together with the same probe and washed together to validate the comparison and to minimize artifacts. Evaluations of signal intensity were determined in a blinded fashion (no knowledge of the genotype). The reproducibility of the results was confirmed independently on at least three separate litters of each knockout colony.
Histology. Sections (5-μm) were deparaffinized, rehydrated in a graded EtOH series, and transferred to distilled water. For morphological assessment of the growth plate, sections were stained with hematoxylin and eosin, or 1% methyl green, and then dehydrated and mounted. For von Kossa staining, the sections were transferred to 1% aqueous silver nitrate solution and exposed for 45 min under a strong light. They were then thrice washed in distilled water, placed in 2.5% sodium thiosulfate (5 min), and thrice washed again in distilled water. Finally, they were counterstained with methyl green, dehydrated in 1-butanol and xylene, and mounted.

Alizarin red S and Alcian blue preparations. Fresh fetuses (ED 18.5) were obtained and the skin, viscera, and adipose tissue were carefully removed. In individual scintillation vials, the fetuses were fixed in 95% EtOH for 5 days, followed by acetone for 2 days to remove the remaining fat and firm up the specimen. After this, the fetuses were stained for 3 days in 10 ml of freshly prepared staining solution at 37°C (1 volume 0.3% Alcian blue 8GS in 70% EtOH-1 volume 0.1% Alizarin red S in 95% EtOH-1 volume acetic acid-17 volumes 70% EtOH). They were then washed in distilled water and immersed in 1% aqueous KOH until the fetal skeleton was clearly visible through the surrounding tissue (~12–48 h). They were cleared in 1% KOH containing increasing concentrations (20, 50, and 80%) of glycerin (7–10 days at each step). Finally, they were transferred into 100% glycerin for permanent storage.

Statistical analysis. Data were analyzed using SYSTAT 5.2.1 for Macintosh (SYSTAT, Evanston, IL). ANOVA was used for the initial analysis; Tukey’s test was used to determine which pairs of means differed significantly from each other. Two-tailed probabilities are reported, and all data are presented as means ± SE.

RESULTS

Maternal effects of calcitonin/CGRPα deletion. Possible maternal effects of calcitonin/CGRPα deletion were examined by comparing pregnant and nonpregnant Ct/Cgrp+/− and Ct/Cgrp null mothers. Ct/Cgrp+/− and Ct/Cgrp null mothers mated readily and conceived with the same frequency, but the number of viable fetuses in utero of Ct/Cgrp null mothers was significantly lower than the number of Ct/Cgrp+/− females when assessed on ED 17.5 or 18.5 at C section (Fig. 1A). Resorption sacs were infrequent and no different in number between litters from Ct/Cgrp+/− and Ct/Cgrp null mothers, indicating that the difference in fetal number developed before the embryonic stage. In contrast, although the trend to lower numbers persisted after birth, the difference was not statistically significant by the time of weaning (Fig. 1B).

On the day before expected birth, maternal ionized calcium was 1.28 ± 0.02 mmol/l in Ct/Cgrp+/− and 1.29 ± 0.02 mmol/l in Ct/Cgrp+/− null dams (P = not significant). These values were not different from the wt or corresponding non-pregnant values (1.28 ± 0.02 mmol/l in Ct/Cgrp+/− and 1.30 ± 0.02 mmol/l in Ct/Cgrp+/− null), indicating that maternal absence of Ct/Cgrp did not impair the mother’s ability to maintain her calcium concentration during the time frame when calcium transfer across the placenta is at its peak. In contrast to the stability of calcium across genotypes and during pregnancy, maternal magnesium was reduced during pregnancy in Ct/Cgrp null mothers. Maternal serum magnesium was 1.17 ± 0.08 mmol/l in nonpregnant Ct/Cgrp null mothers and was reduced to 0.98 ± 0.07 mmol/l in pregnant Ct/Cgrp null mothers on the day before expected birth (P < 0.001). In contrast, serum magnesium was not different between the pregnant and nonpregnant state in Cgrp+/− mothers (1.17 ± 0.05 and 1.14 ± 0.04 mmol/l, respectively).

Placental calcium transfer. To definitively determine whether calcitonin and CGRPα are required by the fetus to maintain placental calcium transfer, we measured the amount of 45Ca transferred to each fetus within 5 min (normalized by 51Cr). In Ct/Cgrp null fetuses obtained from Ct/Cgrp+/− mothers, placental calcium transfer occurred at a rate that was indistinguishable from that of Ct/Cgrp+/− and wt siblings (Fig. 2A). We further assessed placental calcium transfer in fetuses of Ct/Cgrp null mothers to determine whether maternal absence of calcitonin and CGRPα, or the combined absence of maternal and fetal calcitonin and CGRPα, would alter the rate of placental calcium transfer. No difference in the relative rate of 45Ca transfer was noted between Ct/Cgrp+/− and Ct/Cgrp null fetuses of Ct/Cgrp null mothers (Fig. 2B), and the transfer rate was the same as in fetuses of Ct/Cgrp+/− mothers. Thus placental calcium transfer was normal regardless of whether the fetus, the mother, or both lacked calcitonin and CGRPα.

Serum and amniotic fluid mineral physiology. Fetal lack of calcitonin and CGRPα did not alter fetal regulation of the blood calcium, as indicated by the normal ionized calcium and fetal-maternal calcium gradient in wt, Ct/Cgrp+/−, and Ct/Cgrp null fetuses obtained from Ct/Cgrp+/− null dams (Fig. 3A). Furthermore, fetal blood calcium regulation was also unaffected by maternal absence of calcitonin and CGRPα, or combined maternal and fetal absence of calcitonin and CGRPα, because Ct/Cgrp+/− and Ct/Cgrp null fetuses had the same ionized calcium level regardless of whether the mother was Ct/Cgrp+/− or Ct/Cgrp null (Fig. 3, A and B). In contrast to the normal ionized calcium levels, serum magnesium concentrations were significantly reduced in Ct/Cgrp null fetuses both when the mother was Ct/Cgrp+/− (Fig. 3C) and when she was Ct/Cgrp null (Fig. 3D). Serum phosphorus levels trended lower in Ct/Cgrp null fetuses but were not significantly different from those of their littersmates in litters obtained from Ct/Cgrp+/− and Ct/Cgrp null dams (Fig. 3, E and F).

In contrast to the altered serum magnesium concentrations in Ct/Cgrp null fetuses, there were no significant differences in the concentrations of magnesium, calcium, or phosphorus in amniotic fluid (Table 1).
Serum PTH was not significantly different among the genotypes, but the mean values trended slightly higher from wt to null (Fig. 4).

Serum calcitonin was measured in fetuses obtained from \( Ct/Cgrp^{+/+} \) mothers (A) and \( Ct/Cgrp \) null mothers (B) and expressed as a % of heterozygous (HET) fetal mean. Values are means ± SE. Nos. of fetuses are indicated in parentheses; wt, wild-type.

Serum PTH was not significantly different among the genotypes, but the mean values trended slightly higher from wt to null (Fig. 4).

Serum calcitonin was measured in fetuses obtained from both \( Ct/Cgrp^{+/+} \) and \( Ct/Cgrp \) null mothers. Within offspring obtained from \( Ct/Cgrp^{+/+} \) mothers, calcitonin dropped in a step-wise fashion from wt to \( Ct/Cgrp^{+/+} \) to \( Ct/Cgrp \) null (undetectable) (Fig. 5A). Similar results were obtained in fetuses from \( Ct/Cgrp \) null mothers, confirming that absence of maternal calcitonin did not affect the fetal calcitonin level (Fig. 5B). Furthermore, fetal calcitonin was undetectable in null fetuses obtained from both \( Ct/Cgrp^{+/+} \) and \( Ct/Cgrp \) null mothers, confirming that calcitonin cannot cross the placenta from mother to fetus in appreciable amounts. The serum calcitonin level was also undetectable in \( Ct/Cgrp \) null mothers, confirming that the gene deletion had eliminated calcitonin. Although the stated detection limit of the assay is 2 pg/ml in rat serum, it is likely that the level of ~8 pg/ml represents a true zero for this assay in mouse serum under these experimental conditions.

Skeletal morphology and mineral content. The fetal skeleton is intimately involved in calcium metabolism, and it mineralizes during late gestation. We have previously demonstrated that it is possible for placental calcium transfer to be normal but the skeleton not accrete mineral at a normal rate (24). Therefore, the fetal skeleton was examined to determine whether lack of calcitonin and CGRPa altered skeletal development, skeletal mineral content, or gene expression within the growth plate. Furthermore, the skeletons were examined to determine whether the increased bone mass observed in adult

Table 1. Amniotic fluid mineral concentrations

<table>
<thead>
<tr>
<th></th>
<th>Calcium</th>
<th>Magnesium</th>
<th>Phosphorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>1.73 ± 0.23 (16)</td>
<td>1.15 ± 0.09 (12)</td>
<td>2.3 ± 0.2 (12)</td>
</tr>
<tr>
<td>( Ct/Cgrp^{+/+} )</td>
<td>1.27 ± 0.17 (30)</td>
<td>1.13 ± 0.06 (23)</td>
<td>1.9 ± 0.2 (20)</td>
</tr>
<tr>
<td>( Ct/Cgrp ) null</td>
<td>1.29 ± 0.21 (15)</td>
<td>1.13 ± 0.07 (15)</td>
<td>2.2 ± 0.2 (13)</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed in mmol/l. wt. Wild-type; \( Ct/Cgrp^{+/+} \), heterozygous calcitonin/calcitonin gene-related peptide (CGRP)-α; \( Ct/Cgrp \) null, CGRPa knockout animals. Nos. of fetuses are indicated in parentheses.
been stained with Alcian blue (for cartilage) and Alizarin red S (for mineralized bone). The fetal skeletons exhibited no detectable abnormalities, including normal length and morphology of the long bones in the appendicular skeleton (Fig. 6, a and b). The relative distribution of mineral also appeared to be normal.

The mineral content of fetal skeletons was determined by obtaining the ash weights of fetuses (which represent largely mineral), and then assaying the ash to determine the calcium and magnesium content. No differences were found in ash weights of fetuses of all of the genotypes, whether obtained from Ct/Cgrp+/- or Ct/Cgrp null mothers (Table 2). This finding confirms the qualitative finding (from skeletal preparations stained with Alizarin red S) that the skeletal mineral content was unaltered by absence of calcitonin and CGRPα.

The ash was further analyzed by atomic absorption spectroscopy to determine its calcium and magnesium content (Table 2). Results were calculated in milligrams of calcium or magnesium per gram of ash and were normalized to the mean heterozygote value for each litter. The calcium content of the fetal skeletons did not differ among the genotypes, whether obtained from Ct/Cgrp+/- or Ct/Cgrp null mothers (Table 2). This finding confirms the qualitative finding (from skeletal preparations stained with Alizarin red S) that the skeletal mineral content was unaltered by absence of calcitonin and CGRPα.

The mineral content of fetal skeletons was determined by obtaining the ash weights of fetuses (which represent largely mineral), and then assaying the ash to determine the calcium and magnesium content. No differences were found in ash weights of fetuses of all of the genotypes, whether obtained from Ct/Cgrp+/- or Ct/Cgrp null mothers (Table 2). This finding confirms the qualitative finding (from skeletal preparations stained with Alizarin red S) that the skeletal mineral content was unaltered by absence of calcitonin and CGRPα.

The growth plates and long bones were further examined by

Growth plate morphology and gene expression. The growth plates were examined histologically, and the distribution of mineral within the long bones was examined semiquantitatively using the von Kossa method. In this method, silver displaces calcium to create black silver phosphate and silver carbonate complexes; because calcium is the only known cation that binds to these insoluble anions in organic tissue, the method is considered to be sufficiently specific for calcium (7, 37). With this method, the mineral present in Ct/Cgrp null growth plates appeared to be distributed normally compared with wt siblings (Fig. 6, c and d). In addition, endochondral bone formation appeared to be normal, as evidenced by the length of the growth plate, chondrocyte morphology from the proliferative to the hypertrophic zones, periosteal thickness, and the lengths of the long bones.

The growth plates and long bones were further examined by in situ hybridization to determine whether the expression of...
nonviable or resorbed gestational sacs. It was maternal loss of calcitonin and CGRP that was associated with the reduction in the number of viable fetuses; in contrast, fetal loss of Ct/Cgrp did not affect the number of viable fetuses, because Ct/Cgrp null fetuses were present in the expected Mendelian ratios in both Ct/Cgrp/H11001/H11002 and Ct/Cgrp null uteri. These findings may have relevance to previous evidence that calcitonin is expressed in the endometrium during the time of implantation (31) and that calcitonin may be required for the blastocyst to implant (42). The decrease in fetal number appeared to persist into the neonatal period but was not statistically significant; maternal culling of the litter after delivery accounts in large part for why the postnatal litter size is more variable than the number of viable fetuses in vitro.

We noted no difference in skeletal ash weight or skeletal calcium content in fetal mice, indicating that lack of calcitonin and CGRPα in either the mother or the fetus did not impair the amount of calcium accreted by the skeleton or total bone mass at the end of gestation. Furthermore, we observed no abnormality in endochondral bone formation, including morphology and gene expression within the growth plates of the fetal long bones. These findings compare with the observation of Hoff et al. (18) that Ct/Cgrp null mice had a progressive increase in bone mass that became apparent between 1 and 3 mo of age (18), and the more recent finding of Dacquin et al. (12) that heterozygous ablation of the calcitonin receptor also leads to a higher bone mass. Our findings confirm that the increase in bone mass does not occur before birth in the Ct/Cgrp null animal.
decrease in fetal serum magnesium from wt to Ct/Cgrp

Fig. 7. Placental histology and gene expression. Placentas of wt (top) and Ct/Cgrp null (−/−) fetuses (bottom). Overall morphology of placentas was unchanged between wt (a) and Ct/Cgrp null (b), including relative distribution and morphology of intraplacental yolk sac (arrows) and labyrinthine trophoblasts (L). At higher magnification (b and f), bilayered structure of intraplacental yolk sac can be visualized, with parietal cells overlying Reichert’s membrane (arrows) and the columnar cells opposite (arrowheads). In situ hybridization studies (c, d, g, and h). Representative dark-field images are shown of calbindin-D9k (c and g) and Ca\(^{2+}\)-ATPase (d and h). Both mRNAs are most intensely expressed in the intraplacental yolk sac (arrows), with less intense expression observed in the labyrinthine trophoblasts (L). No difference in relative expression of either mRNA was observed between wt and Ct/Cgrp null placentas.
may contribute to the regulation of magnesium metabolism in the adult. A number of investigators have demonstrated that calcitonin stimulates the conservation of both magnesium and calcium by the renal tubules (10, 11, 13), but those observations may represent pharmacological and not physiological effects of calcitonin. We observed no alteration in the apparent renal excretion of calcium, magnesium, and phosphorus in Ctgpr null fetuses.

In summary, we examined several indexes of mineral metabolism in this study of Ctgpr ablation in fetal mice. We found that the serum magnesium level and skeletal magnesium content were reduced in Ctgpr null fetuses but that parameters of calcium metabolism (serum calcium, placental calcium transfer, and skeletal calcium content) were unaltered. Fetal weight, growth plate morphology, and gene expression within the growth plates were normal in Ctgpr null fetuses, in contrast to the increased bone mass that was observed in Ctgpr null adults. Placental morphology, weight, and gene expression were also unaltered. Ctgpr null mothers had pregnancies with significantly fewer viable fetuses, but this difference was not significant after maternal culling of the litters in the neonatal period. Taken as a whole, these results indicate that calcitonin and CgRPa are required for some determinant of gestational litter size (possibly implantation of the blastocyst) and for normal fetal magnesium homeostasis. Calcitonin and CgRPαs are not essential for later survival and development of the fetus, and they are not required to maintain placental calcium transfer, fetal calcium homeostasis, and calcium accretion by the fetal skeleton.

ACKNOWLEDGMENTS

The additional technical support of Linda L. Chafe, Mandy L. Woodland, and Claude Mercer is acknowledged. Part of the work described in this paper was done to fulfill the MSc thesis requirements for K. R. McDonald and garnered her the Mary Pater Graduate Studies Award for Excellence in Cancer Research.

Current address of J. K. Friel is Department of Human Nutritional Sciences, Faculty of Human Ecology, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada.

GRANTS

This study was supported by operating grants and a five-year Scholarship (New Investigator Award) from the Canadian Institutes for Health Research (formerly Medical Research Council of Canada), and by funds from the Research and Development Committee, the Medical Research Foundation, and the Discipline of Medicine, all in the Faculty of Medicine at Memorial University of Newfoundland (all to C. S. Kovacs).

REFERENCES

28. Kovacs CS, Lanske B, Hunzelson JL, Guo J, Karaplis AC, and Kronenberg HM. Parathyroid hormone-related peptide (PTHrP) regu-
lates fetal-placental calcium transport through a receptor distinct from the PTH/PTHrP receptor. *Proc Natl Acad Sci USA* 93: 15233–15238, 1996.


